DESCRIPTION

DEPROTECTION OF RNA

This Application claims the benefit of Laurent Bellon, et al., U.S Provisional Application 60/061,321, entitled "Deprotection of RNA", filed October 2, 1997. This application is hereby incorporated herein by reference in its entirety, including any drawings and figures.

Background of the Invention

This invention relates to the synthesis, deprotection, and purification of RNA.

Generally, RNA molecules are chemically synthesized and purified by methodologies based on the use of tetrazole to activate the RNA phosphoramidite, ethanolic-NH_4OH to remove the exocyclic amino protecting groups, tetra-*n*-butylammonium fluoride (TBAF) to remove the 2'-*OH* alkylsilyl protecting groups, and gel purification and analysis of the deprotected RNA. Examples of chemical synthesis, deprotection, purification and analysis procedures for RNA are provided by Usman et al., 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al. Nucleic Acids Res.* 1990, *18*, 5433-5341; Perreault *et al. Biochemistry* 1991, *30* 4020-4025; Slim and Gait *Nucleic Acids Res.* 1991, *19*, 1183-1188. All the above noted references are all hereby incorporated by reference herein.

The deprotection process commonly involves the deprotection of the exocyclic amino protecting groups by NH_4OH, which is time consuming (6-24 h) and inefficient. This step is then followed by treatment with TBAF to facilitate the removal of alkylsilyl protecting groups, which again is time consuming and not very effective in achieving efficient deprotection.

A recent modification of this two-step strategy for oligoribonucleotide deprotection has been reported by Wincott *et al.*, (*Nucleic Acids Res.*, 1995, 23, 2677-2784) and by Vinayak *et al.*, (*Nucleic Acids Symposium series*, 1995. 33, 123-125). The optimized conditions make use of aqueous methylamine at 65°C for 15 minutes in place of the ammonium hydroxide cocktail to remove exocyclic amino protecting groups while the desilylation treatment needed to remove the 2'-*OH* alkylsilyl protecting groups utilizes a mixture of triethylamine trihydrogen fluoride (TEA.3HF), N-methyl-pyrrolidinone and triethylamine at 65°C for 90 minutes, thereby replacing tetrabutyl ammonium fluoride.

Stinchcomb *et al.*, International PCT Publication No. *WO 95/23225* describe a process for one pot deprotection of RNA. On page 73, it states that:

"In an attempt to minimize the time required for deprotection and to simplify the process of deprotection of RNA synthesized on a large scale, applicant describes a one pot deprotection protocol... According to this protocol, anhydrous methylamine is used in place of aqueous methyl amine. Base deprotection is carried out at 65 °C for 15 minutes and the reaction is allowed to cool for 10 min. Deprotection of 2'-hydroxyl groups is then carried out in the same container for 90 minutes in a TEA•3HF reagent. The reaction is quenched with 16 mM TEAB solution."

Summary of the Invention

This invention concerns a one-pot process for the deprotection of RNA molecules. This invention features a novel method for the removal of protecting groups from the nucleic acid base and 2'-*OH* groups, which accelerates the process for generating synthetic RNA in a high throughput manner (*e.g.*, in a 96 well format).

Chemical synthesis of RNA is generally accomplished using a traditional column format on a RNA synthesizer where only one oligoribonucleotide is synthesized at a time. Simultaneous synthesis of more than one RNA molecule in a time efficient manner requires alternate methods to the traditional column format, such as synthesis in a 96 well plate format where up to 96 RNA molecules can be synthesized at the same time. To expedite this process of simultaneous synthesis of multiple RNA molecules, it is important to accelerate some of the time consuming processes such as the deprotection of RNA following synthesis (*i.e.* removal of base protecting group, such as the exocyclic amino protecting group and the phosphate protecting groups and the removal of 2'-*OH* protecting groups, such as the tButylDiMethylSilyl). In a preferred embodiment, the invention features a one-pot process for rapid deprotection of RNA.

Stinchcomb *et al., supra* described a one-pot protocol for RNA deprotection using anhydrous methylamine and triethylamine trihydrogen fluoride. This procedure involves the use of an anhydrous solution of base such as a 33% methylamine in absolute ethanol followed by neat triethylamine trihydrofluoride to effectively deprotect oligoribonucleotides in a one-pot fashion. However such a protocol may be cumbersome for deprotection of RNA synthesized on a plate format, such as a 96 well plate, because it may be necessary to separate the solid-support from the partially deprotected RNA prior to the 2'-hydroxyl deprotection. Also, since the methylamine solution used is anhydrous, it may be difficult to solubilize the negatively charged

oligoribonucleotides obtained after basic treatment. So, in a first aspect the invention features the use of a 1:1 mixture of the ethanolic methylamine solution and a polar additive, such as dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), methanol, hexamethylphosphoramide (HMPA), 1-methyl-2-pyrrolidinone (NMP), 2-methoxyethyl ether (glyme) or the like. More specifically, dimethylsufoxide is used to partially deprotect oligoribonucleotides (Figure 2). A comparison of the one pot and two pot deprotection methods are outlined and demonstrated in Figure 3.

This invention also concerns a rapid (high through-put) deprotection of RNA in a 96-well plate format. More specifically rapid deprotection of enzymatic RNA molecules in greater than microgram quantities with high biological activity is featured. It has been determined that the recovery of enzymatically active RNA in high yield and quantity is dependent upon certain critical steps used during its deprotection.

In a preferred embodiment, the invention features a process for one-pot deprotection of RNA molecules comprising protecting groups, comprising the steps of:. a) contacting the RNA with a mixture of anhydrous alkylamine (where alkyl can be branched or unbranched, ethyl, propyl or butyl and is preferably methyl, *e.g.*, methylamine), trialkylamine (where alkyl can be branched or unbranched, methyl, propyl or butyl and is preferably ethyl, *e.g.*, ethylamine) and dimethylsulfoxide, preferably in a 10:3:13, or 1:0.3:1 proportion at temperature 20-30 °C for about 30-100 minutes, preferably 90 minutes, to remove the exocyclic amino (base) protecting groups and the phosphate protecting group (*e.g.*, 2-cyanoethyl) (*vs* 4-20 h at 55-65 °C using NH_4OH/EtOH or NH_3/EtOH, or 10-15 min at 65°C using 40% aqueous methylamine) under conditions suitable for partial deprotection of the RNA; b) contacting the partially deprotected RNA with anhydrous triethylamine•hydrogen fluoride (3HF•TEA) and heating at about 50-70 °C, preferably at 65 °C, for about 5-30 min, preferably 15 min to remove the 2'-hydroxyl protecting group (*vs* 8 - 24 h using TBAF, or TEA•3HF for 24 h (Gasparutto *et al. Nucleic Acids Res.* 1992, 20, 5159-5166) (Other alkylamine•HF complexes may also be used, *e.g.* trimethylamine or diisopropylethylamine) under conditions suitable for the complete deprotection of the RNA. The reaction can then be quenched by using aqueous ammonium bicarbonate (1.4 M). Although some other buffers can be used to quench the desilylation reaction (*i.e.* triethylammonium bicarbonate, ammonium acetate), the ammonium bicarbonate buffer is perfectly suited to retain the 5'-*O*-dimethoxytrityl group at the 5'-end of the

oligoribonucleotide thereby facilitating a reverse phase-based solid-phase extraction purification protocol.

By "one-pot" deprotection is meant that the process of deprotection RNA is carried out in one container instead of multiple containers as in two-pot deprotection.

In another preferred embodiment, the invention features a process for one pot deprotection of RNA molecules comprising protecting groups, comprising the steps of: a) contacting the RNA with a mixture of anhydrous alkylamine (where alkyl can be branched or unbranched, ethyl, propyl or butyl and is preferably methyl, *e.g.*, methylamine), and dimethylsulfoxide, preferably in a 1:1 proportion at 20-30 °C temperature for about 30-100 minutes, preferably 90 minutes, to remove the exocyclic amino (base) protecting groups and the phosphate protecting group (*e.g.*, 2-cyanoethyl) (*vs* 4-20 h at 55-65 °C using NH_4OH/EtOH or NH_3/EtOH, or 10-15 min at 65°C using 40% aqueous methylamine) under conditions suitable for partial deprotection of the RNA; b) contacting the partially deprotected RNA with anhydrous triethylamine•hydrogen fluoride (3HF•TEA) and heating at about 50-70 °C, preferably at 65 °C, for about 5-30 min, preferably 15 min to remove the 2'-hydroxyl protecting group (Other alkylamine•HF complexes may also be used, *e.g.* trimethylamine or diisopropylethylamine) under conditions suitable for the complete deprotection of the RNA. The reaction can then be quenched by using aqueous ammonium bicarbonate (1.4 M). Although some other buffers can be used to quench the desilylation reaction (*i.e.* triethylammonium bicarbonate, ammonium acetate), the ammonium bicarbonate buffer is perfectly suited to retain the 5'-*O*-dimethoxytrityl group at the 5'-end of the oligoribonucleotide thereby facilitating a reverse phase-based solid-phase extraction purification protocol.

In another aspect the invention features a process for RNA deprotection where the exocyclic amino and phosphate deprotection reaction is performed with the ethanolic methylamine solution at room temperature for about 90 min or at 65°C for 15 min or at 45°C for 30 min or at 35°C for 60 min.

In a preferred embodiment, the process for deprotection of RNA of the present invention is used to deprotect a ribozyme synthesized using a column format as described in (Scaringe *et al.*, supra; Wicott *et al.*, *supra*).

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings:

Figure 1 shows the secondary structure model for seven different classes of enzymatic nucleic acid molecules. Arrow indicates the site of cleavage. -------- indicate the target sequence. Lines interspersed with dots are meant to indicate tertiary interactions. - is meant to indicate base-paired interaction. **Group I Intron:** P1-P9.0 represent various stem-loop structures (Cech *et al.*, 1994, *Nature Struc. Bio.*, 1, 273). **RNase P (M1RNA):** EGS represents external guide sequence (Forster *et al.*, 1990, *Science*, 249, 783; Pace *et al.*, 1990, *J. Biol. Chem.*, 265, 3587). **Group II Intron:** 5'SS means 5' splice site; 3'SS means 3'-splice site; IBS means intron binding site; EBS means exon binding site (Pyle *et al.*, 1994, *Biochemistry*, 33, 2716). **VS RNA:** I-VI are meant to indicate six stem-loop structures; shaded regions are meant to indicate tertiary interaction (Collins, International PCT Publication No. WO 96/19577). **HDV Ribozyme:** : I-IV are meant to indicate four stem-loop structures (Been *et al.*, US Patent No. 5,625,047). **Hammerhead Ribozyme:** : I-III are meant to indicate three stem-loop structures; stems I-III can be of any length and may be symmetrical or asymmetrical (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527). **Hairpin Ribozyme:** Helix 1, 4 and 5 can be of any length; Helix 2 is between 3 and 8 base-pairs long; Y is a pyrimidine; Helix 2 (H2) is provided with a least 4 base pairs (*i.e.*, n is 1, 2, 3 or 4) and helix 5 can be optionally provided of length 2 or more bases (preferably 3 - 20 bases, *i.e.*, m is from 1 - 20 or more). Helix 2 and helix 5 may be covalently linked by one or more bases (*i.e.*, r is ≥ 1 base). Helix 1, 4 or 5 may also be extended by 2 or more base pairs (*e.g.*, 4 - 20 base pairs) to stabilize the ribozyme structure, and preferably is a protein binding site. In each instance, each N and N' independently is any normal or modified base and each dash represents a potential base-pairing interaction. These nucleotides may be modified at the sugar, base or phosphate. Complete base-pairing is not required in the helices, but is preferred. Helix 1 and 4 can be of any size (*i.e.*, o and p is each independently from 0 to any number, *e.g.*, 20) as long as some base-pairing is maintained. Essential bases are shown as specific bases in the structure, but those in

the art will recognize that one or more may be modified chemically (abasic, base, sugar and/or phosphate modifications) or replaced with another base without significant effect. Helix 4 can be formed from two separate molecules, *i.e.*, without a connecting loop. The connecting loop when present may be a ribonucleotide with or without modifications to its base, sugar or phosphate. "q" is ≥ 2 bases. The connecting loop can also be replaced with a non-nucleotide linker molecule. H refers to bases A, U, or C. Y refers to pyrimidine bases. "_____" refers to a covalent bond. (Burke *et al.*, 1996, *Nucleic Acids & Mol. Biol.*, 10, 129; Chowrira *et al.*, US Patent No. 5,631,359).Figure 2a is a diagrammatic representation of the hammerhead ribozyme domain known in the art; Figure 2b is a diagrammatic representation of the hammerhead ribozyme as divided by Uhlenbeck (1987, *Nature*, 327, 596-600) into a substrate and enzyme portion; Figure 2c is a similar diagram showing the hammerhead divided by Haseloff and Gerlach (1988, *Nature* , 334, 585-591) into two portions; and Figure 2d is a similar diagram showing the hammerhead divided by Jeffries and Symons (1989, *Nucl. Acids. Res.*, 17, 1371-1371) into two portions.

Figure 2 is a is a schematic representation of a one pot deprotection of RNA synthesized using RNA phosphoramidite chemistry.

Figure 3 is a comparison of a one-pot and a two-pot process for deprotection of RNA.

Figure 4 shows the results of a one-pot deprotection with different polar organic reagents.

Synthesis and purification of RNA

By "RNA" or "oligoribonucleotides" as used herein is meant a molecule having one or more ribonucleotides. The RNA can be single, double or multiple stranded and may comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

RNA molecules can be chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and

phosphoramidites at the 3'-end. Small scale synthesis were conducted on a 394 Applied Biosystems, Inc. synthesizer using a modified 2.5 µmol scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-*O*-methylated nucleotides. Table I outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163 µL of 0.1 M = 16.3 µmol) of phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole (238 µL of 0.25 M = 59.5 µmol) relative to polymer-bound 5'-hydroxyl was used in each coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, were 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer: detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I_2, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile was used directly from the reagent bottle. *S*-Ethyl tetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical, Inc.

Deprotection of the RNA was performed according to the present invention. RNAs are purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Stinchcomb *et al.*, International PCT Publication No. WO 95/23225, the totality of which is hereby incorporated herein by reference) and are resuspended in water.

Enzymatic RNA molecules:

The enzymatic RNA molecule is a nucleic acid molecule comprising at least one ribonucleotide. Enzymatic RNA molecule is able to intramolecularly or intermolecularly cleave RNA or DNA and thereby inactivate a target RNA or DNA molecule. The enzymatic RNA acid molecule that has complementarity in a substrate binding region to a specified gene target, also has an enzymatic activity that specifically cleaves RNA or DNA in that target. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA or DNA to allow the cleavage to occur. 100% Complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention. The nucleic acids may be modified at the base, sugar, and/or phosphate groups.

The term enzymatic RNA acid is used interchangeably with phrases such as ribozymes, enzymatic nucleic acid, catalytic RNA, enzymatic RNA, nucleozyme, RNA enzyme, endoribonuclease, minizyme, leadzyme, oligozyme and the like.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with other RNA sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

RNA molecules having an endonuclease enzymatic activity are able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. Such enzymatic RNA molecules can be targeted to virtually any RNA transcript, and efficient cleavage achieved *in vitro* (Zaug *et al.*, 324, *Nature* 429 1986 ; Kim et al., 84 *Proc. Natl. Acad. Sci. USA* 8788, 1987; Haseloff and Gerlach, 334 *Nature* 585, 1988; Cech, 260 *JAMA* 3030, 1988; and Jefferies et al., 17 *Nucleic Acids Research* 1371, 1989).

Because of their sequence-specificity, *trans*-cleaving ribozymes show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Ribozymes can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the mRNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited.

Seven basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. Figure 1 summarizes some of the characteristics of these ribozymes. In general, enzymatic RNA act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is

released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over other technologies, since the effective concentration of ribozyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base-pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

In one aspect enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif (Figure 1), but may also be formed in the motif of a hepatitis delta virus (HDV), group I intron, RNaseP RNA (in association with an external guide sequence) or *Neurospora* VS RNA (Figure 1). Examples of such hammerhead motifs are described by Rossi *et al.*, 1992, *Aids Research and Human Retroviruses* 8, 183; Usman *et al.*, 1996, *Curr. Op. Struct. Biol.*, 1, 527; of hairpin motifs by Hampel *et al.*, EP 0360257; Hampel and Tritz, 1989 *Biochemistry* 28, 4929; and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; Chowrira *et al.*, US Patent No. 5,631,359; an example of the hepatitis delta virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; Been *et al.*, US Patent No. 5,625,047; of the RNaseP motif by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990 *Science* 249, 783; *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Guo and Collins, 1995 *EMBO J.* 14, 368) and of the Group I intron by Zaug *et al.*, 1986, Nature, 324, 429; Cech *et al.*, U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule with endonuclease activity of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA and that it have nucleotide sequences within or surrounding that substrate binding site which

impart an RNA cleaving activity to the molecule. The length of the binding site varies for different ribozyme motifs, and a person skilled in the art will recognize that to achieve an optimal ribozyme activity the length of the binding arm should be of sufficient length to form a stable interaction with the target nucleic acid sequence.

Catalytic activity of the ribozymes described in the instant invention can be optimized as described by Draper et al., *supra*. The details will not be repeated here, but include altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases and/or enhance their enzymatic activity (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken et al., 1991 *Science* 253, 314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al., supra;* all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of enzymatic RNA molecules). Modifications which enhance their efficacy in cells, and removal of bases from stem loop structures to shorten RNA synthesis times and reduce chemical requirements are desired. (All these publications are hereby incorporated by reference herein).

There are several examples in the art describing sugar and phosphate modifications that can be introduced into enzymatic nucleic acid molecules without significantly effecting catalysis and with significant enhancement in their nuclease stability and efficacy. Ribozymes are modified to enhance stability and/or enhance catalytic activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34; Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996 *Biochemistry* 35, 14090). Sugar modification of enzymatic nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No. WO 92/07065; Perrault *et al. Nature* 1990, *344*, 565-568; Pieken *et al. Science* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *US Patent* No. 5,334,711 and Beigelman *et al.*, 1995 *J. Biol. Chem.* 270, 25702; all of the references are hereby incorporated in their totality by reference herein).

Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes without inhibiting catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid catalysts of the instant invention.

Nucleic acid catalysts having chemical modifications which maintain or enhance enzymatic activity are provided. Such nucleic acid is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in a cell and/or *in vivo* the activity may not be significantly lowered. As exemplified herein such ribozymes are useful in a cell and/or *in vivo* even if activity over all is reduced 10 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Such ribozymes herein are said to "maintain" the enzymatic activity on all RNA ribozyme.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a sugar moiety. Nucleotide generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other ; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; all hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art and has recently been summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into enzymatic nucleic acids without significantly effecting their catalytic activity include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine) and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used within the catalytic core of the enzyme and/or in the substrate-binding regions.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

Various modifications to ribozyme structure can be made to enhance the utility of ribozymes. Such modifications will enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such ribozymes to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells..

Deprotection of RNA

For high throughput chemical synthesis of oligoribonucleotides, it is important that the two main steps involved in the deprotection of oligoribonucleotides (i.e. aqueous basic treatment to remove exocyclic amino protecting groups and phosphate protecting groups and fluoride treatment to remove the 2'-*OH* alkylsilyl protecting groups such as the tButylDiMethylSilyl) are condensed.

Stinchcomb *et al.*, *supra* describe a time-efficient (~ 2 hrs) one-pot deprotection protocol based on anhydrous methylamine and triethylamine trihydrogen fluoride. Since it has recently been reported that water contamination during fluoride treatment may be detrimental to the efficiency of the desilylation reaction (Hogrefe et al, Nucleic Acids Res. (1993), 21 4739-4741), it is necessary to use an anhydrous solution of base such as a 33% methylamine in absolute ethanol followed by neat triethylamine trihydrofluoride to effectively deprotect oligoribonucleotides in a one-pot fashion. However it may be cumbersome to apply such a protocol to plate format deprotection where the solid-support is preferentially separated from the partially deprotected oligoribonucleotides prior to the 2'-hydroxyl deprotection. Indeed, because the methylamine solution used is anhydrous, it may not be suitable to solubilize the negatively charged oligoribonucleotides obtained after basic treatment. Therefore, applicant investigated a 1:1 mixture of the ethanolic methylamine solution and different polar additives such as dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF), methanol, hexamethylphosphoramide (HMPA), 1-methyl-2-pyrrolidinone (NMP) or 2-methoxyethyl ether (glyme). Of all these additives, dimethylsufoxide is capable of efficiently solubilizing partially deprotected oligoribonucleotides (figure 4). A

comparison of the one pot and two pot deprotection methods are outlined and demonstrated in figure 3.

Examples

The following are non-limiting examples showing the deprotection of RNA.

Example 1: Deprotection of Ribozyme in a 96 Well Plate

A ribozyme sequence (200nmole) was synthesized as described herein on a polystyrene solid support in a well of a 96 well plate. A 10:3:13 mixture (800 µL) of anhydrous methylamine (308µL), triethylamine (92µL) and dimethylsulfoxide (DMSO) (400 µL) was prepared of which half (400 µL) was added to the well and incubated at room temperature for 45 minutes. Following the reaction the solution was replaced with the remaining 400 µL and incubated as before. At the end of the reaction, the solid support was filtered off, all 800 µL of MA/TEA/DMSO solution was collected together and 100 µL of TEA.3HF was added. The reaction was then heated at 65°C for 15 minutes and then cooled to room temperature. The solution was then quenched with aqueous $NH_4^+HCO_3^-$ (1mL) (see Figure 2). HPLC chromatography of the reaction mixture afforded 32 O. D.$u_{260\ nm}$ of which 46% was full length ribozyme.

Example 2: Column Deprotection of Ribozyme

A ribozyme was synthesized using the column format as described herein. The polystyrene solid-support with protected oligoribonucleotide or modified oligoribonucleotide (200 nmole) was transferred into a glass vial equipped with a screw cap. A 10:3:13 mixture of anhydrous methylamine (308 µL), triethylamine (92 µL) and dimethylsulfoxide (DMSO) (400 µL) was added followed by vortexing of the glass vial. After allowing the reaction for 1.5 hours, the solid support was filtered off. 100 µL of TEA.3HF was added at room temperature to the vial and the reaction was mixed causing the solution to gel. The reaction was then heated at 65 °C for 15 minutes and then cooled to room temperature. The solution was then quenched with 1.5 M aqueous $NH_4^+HCO_3^-$ (1mL). HPLC chromatography of the reaction mixture afforded 32 O. D.$u_{260\ nm}$ of which 46% was full length ribozyme.

Example 3. Column Deprotection of Ribozyme with anhydrous ethanolic methylamine.

A ribozyme was synthesized using the column format as described herein. The polystyrene solid-support with protected oligoribonucleotide or modified oligoribonucleotide (200 nmole) was transferred into a glass vial equipped with a screw cap. A 1:1 mixture of anhydrous ethanolic methylamine (400 µL) and dimethylsulfoxide (DMSO) (400 µL) was added followed by vortexing of the glass vial. After allowing the reaction for 1.5 hours, the solid support was filtered off. 100 µL of TEA.3HF was added at room temperature to the vial and the reaction was mixed causing the solution to gel. The reaction was then heated at 65 °C for 15 minutes and then cooled to room temperature. The solution was then quenched with 1.5 M aqueous $NH_4^+HCO_3^-$ (1mL). HPLC chromatography of the reaction mixture afforded 32 O. D.$u_{260\ nm}$ of which 46% was full length ribozyme.

Example 4. Large-scale One-Pot Deprotection of Ribozyme.

A ribozyme was synthesized at the 0.5 mmol scale using the column format as described herein. The polystyrene solid-support (24 grs) with protected oligoribonucleotide or modified oligoribonucleotide (500 µmole) was transferred into a 1L Schott bottle equipped with a screw cap. A 1:1.3 mixture of anhydrous ethanolic methylamine (150 mL) and dimethylsulfoxide (DMSO) (200 mL) was added followed by vortexing (200 rpm) of the glass bottle for 1.5 hours. The reaction mixture was then frozen at -70 °C for 30 minutes. 50 mL of neat TEA.3HF was then added at room temperature to the reaction mixture and the reaction was placed in a shaking oven (200 rpm) where it was heated at 65 °C for 60 minutes and subsequently frozen at -70 °C for 30 minutes. The solution was then quenched with 1.5 M aqueous $NH_4^+HCO_3^-$ (200 mL). The reaction mixture was separated from the polystyrene solid-support by filtration on a sintered glass funnel (10-20 µm porosity). U.V. spectrophotometric quantification and HPLC chromatography of the reaction mixture afforded 160,000 O.D.$u_{260\ nm}$ of which 46.4% was full length ribozyme. After allowing the reaction for 1.5 hours, the solid support was filtered off

Other embodiments are within the following claims.

Table I

Table I: 2.5 µmol RNA Synthesis Cycle

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	6.5	163 µL	2.5
S-Ethyl Tetrazole	23.8	238 µL	2.5
Acetic Anhydride	100	233 µL	5 sec
N-Methyl Imidazole	186	233 µL	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

* Wait time does not include contact time during delivery.